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An IκB Homolog Encoded by African Swine Fever Virus Provides a Novel Mechanism for Downregulation of Proinflammatory Cytokine Responses in Host Macrophages

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Cytokines stimulate inflammatory defenses against viral infections. In order to evade host defenses, viruses have developed strategies to counteract antiviral cytokines. African swine fever virus (ASFV) is a large, double-stranded DNA virus that infects macrophages. This study demonstrates that ASFV effectively inhibited phorbol myristic acid-induced synthesis of antiviral, proinflammatory cytokines alpha interferon, tumor necrosis factor alpha, and interleukin-8 in infected macrophages as assessed by enzyme-linked immunosorbent assay and reverse transcriptase PCR. In contrast, levels of mRNA and protein for transforming growth factor β, an anti-inflammatory cytokine, were increased by ASFV infection, suggesting that ASFV-induced inhibition of cytokine synthesis may be limited to cytokines activated by NFκB. An interleukin-8 promoter, containing an NFκB enhancer site, driving expression of a luciferase reporter gene was used to show that NFκB-dependent transcription was inhibited by the virus and by a cloned ASFV gene, A238L. This gene encodes a protein with homology to IκB, the inhibitor of NFκB. Electrophoretic mobility shift assay showed that cells expressing the A238L gene inhibited NFκB binding to DNA. These results suggest that the A238L gene product interacts with NFκB to prevent transcription and downregulate proinflammatory cytokine production. This novel viral evasion strategy encoded in a single IκB-like protein may be capable of inhibiting most macrophage NFκB-dependent antiviral mechanisms and may provide insights into how ASFV causes a fatal hemorrhagic disease of domestic pigs and a persistent infection in the African warthog, which is its natural permissive host.

Cytokines are important mediators of inflammation and regulators of the immune response. The inflammatory response is the first defense against viral infection, rapidly initiated and effectively mounted before the immune response has been established. Induction of cytokine expression is extremely quick, and effector cells are activated locally to promote killing of virally infected cells. In response to this, pathogens have evolved a number of diverse strategies to avoid the host inflammatory response. Large DNA viruses which have a complex genome, such as poxviruses, adenoviruses, and herpesviruses, have genes that are nonessential for replication that can inhibit the activity of cytokines (28). Interestingly, some of these viral proteins have been captured from their host and modified, retaining binding of the natural ligand, but inhibiting the host gene function (2). Viruses that modulate cytokine action include poxviruses that encode secreted forms of receptors for tumor necrosis factor alpha (TNF-α), alpha interferon (IFN-α), and interleukin-1 (IL-1) (15, 29, 30); herpesviruses, cytomegalovirus, and capripox virus, which encode chemokine receptors (1, 6, 21); and adenoviruses that possess intracellular proteins that counteract TNF-α (35).

African swine fever virus (ASFV) is an interesting example of the newly emerging, highly pathogenic group of hemorrhagic viruses (8). Until recently, ASFV existed only as a nonpathogenic, persistent infection of African warthogs and bushpigs, transmitted and maintained by acarine (soft tick) vectors (10). With increased population movement and higher-intensity farming, however, the virus has penetrated the domestic pig population, causing alarming losses in many African countries and becoming endemic in parts of the Iberian peninsula and Sardinia (34). Following transmission, the virus replicates in macrophages, and in the most virulent isolates, death of domestic pigs ensues in 4 to 6 days. In the absence of any basic understanding of resistance and persistence in either the natural or the acarine host, it is hardly surprising that there is no vaccine, and control of the disease is restricted to slaughter and incineration. The key issues of resistance in warthogs, on the one hand, versus lethal hemorrhagic pathology in pigs, on the other, may be best addressed through an elucidation of viral survival strategies. Clues for these may be found in the large double-stranded, linear DNA genome (170 to 180 kb) and in the virus tropism for macrophages. The recent publication of the complete sequence of ASFV (36) has revealed a number of interesting functional reading frames. Some of these proteins are involved in nucleic acid metabolism, prevention or repair of DNA damage, or modification of proteins or are putative membrane-associated or secreted proteins (14, 24, 25, 36). ASFV also encodes a number of proteins potentially able to interfere with the host immune response to the viral infection (36).

The aim of this study was to elucidate how ASFV gene products might impair the cytokine-induced inflammatory defenses of its host cell, the macrophage. ASFV must also counteract the effect of cytokines on its replication in macrophages (11). In this study, the change in expression of protein and mRNA for several porcine proinflammatory cytokines was measured in macrophages after ASFV infection. The analysis showed that secretion and transcription of proinflammatory cytokines were inhibited after ASFV infection. The regulatory elements of the IL-8 gene cloned upstream from a luciferase reporter gene were used to show that the virus inhibited

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NFκB-dependent gene transcription. The involvement of the ASFV gene, A238L, encoding a structural homolog of IκB, was analyzed as a candidate inhibitor of cytokine expression (17). Expression of the viral IκB-A238L alone in cells inhibited transcription from the IL-8 promoter luciferase construct. Electrophoretic mobility shift assay (EMSA) of nuclear extracts from transfected cells showed that A238L also inhibited NFκB binding to its DNA recognition site. Thus, a functionally active, virally encoded IκB activity provides a novel strategy to manipulate host proinflammatory cytokine levels.

MATERIALS AND METHODS

Viruses and cells. ASFV isolates used were Malawi LIL/20/1 (12), OUR T/88-1 (5), and Uganda (13). ASFV was purified from infected bone marrow cell lysates by low-speed centrifugation followed by centrifugation at 25,000 × *g* for 1 h. Pellets were resuspended in phosphate-buffered saline (PBS) and resedimented through a cushion of 25% sucrose. For some experiments, virus was treated with a shortwave UV light for 5 min to inactivate it.

Porcine alveolar macrophages were obtained from the lungs of normal outbred pigs by bronchoalveolar lavage. Cells were allowed to adhere to plastic and then infected immediately with ASFV in alpha minimum essential medium (α-MEM) without serum. The multiplicity of infection of virus to cells was >10:1. After 1 h, the medium was replaced with α-MEM with 10% fetal calf serum or with α-MEM containing 100 nM phorbol myristic acid (PMA) as indicated.

Enzyme-linked immunosorbent assay (ELISA). IFN-α was measured in the supernatants of PMA-stimulated cells with a capture ELISA using two porcine specific anti-IFN-α monoclonal antibodies, K9 and peroxidase-conjugated F17 (16, 22). Plates were coated overnight with anti-porcine IFN-α monoclonal antibody (K9) at 0.8 μg/ml in 50 mM carbonate buffer, pH 9.5. After blocking with 0.5% bovine serum albumin–0.05% Tween 20 in PBS and extensive washing, samples (50 to 200 μl) were incubated in each well for 5 h. Following further extensive washing, the plates were incubated with peroxidase-conjugated anti-porcine F17 monoclonal antibody. After incubation for 2 h and washing, substrate solution *o*-phenylenediamine dihydrochloride (0.4 mg/ml) and hydrogen peroxide (0.03%) in 0.05 M citrate buffer (pH 4.0) was added for 30 min, and the optical density was measured in an ELISA plate reader at 495 nm. TNF-α was similarly measured by ELISA using two antihuman antibodies which cross-reacted with pig TNF-α. Plates were coated with a monoclonal anti-TNF-α antibody and washed extensively, and samples of macrophage supernatants were added. A second rabbit anti-TNF antibody was used for the detection, and signal was visualized with a goat anti-rabbit horseradish peroxidase conjugate. Transforming growth factor β1 (TGF-β1) was measured in the supernatants of cells by antibody capture ELISA using the quantitative Predicta ELISA kit (Genzyme) according to the manufacturer's instructions. The human and porcine TGF-β1 sequences are identical, so that this kit is equally sensitive for both species. Results show the average of three experiments.

RNA isolation and reverse transcriptase PCR. Alveolar macrophages (2×10^7 cells per time point) were treated with PMA with or without ASFV or inactivated ASFV for 0, 2, or 4 h. Total RNA was isolated by scraping cells into denaturing solution (4 M guanidinium isothiocyanate, 25 mM citrate [pH 7.0], 0.1 M 2-mercaptoethanol, 0.5% Sarkosyl) (7). The solution was acidified with 2 M sodium acetate (pH 4.0), and water-saturated phenol and chloroform were added. The RNA was extracted from the aqueous layer, precipitated, and quantitated. Total RNA (10 μg) was reverse transcribed into single-stranded cDNA with avian myeloblastosis virus reverse transcriptase and oligo(dT) primers. Amplification of cytokine DNA was carried out by PCR with the following primers at 0.4 μM derived from porcine sequences in GenBank. TGF-β1 primers were 5′-ATTCG CGGCCAGATTCTGTCCAAGC-3′ and 5′-GGTAGCGCCAGGAATCATTG CTGTA-3′. TNF-α primers were 5′-CTCTTCTGCCTACTGCACTTCGAGG -3′ and 5′-CTGGGAGTAGATGAGGTACAGCCCA-3′. IL-8 primers were 5′-AGCCCGTGTCAACATGACTTCC-3′ and 5′-GAATTGTGTTGGCATCTTT ACTGAG-3′. Primers for IFN-α were 5′-ATGGCCCCAACCTCAGCCTTC-3′ and 5′-TCACTCCTTCTTCCTGAGTCT-3′. DNA was denatured and amplified by cycles of 94°C denaturation for 30 s, 60°C annealing for 30 s, and 72°C extension for 30 s with a 5-min final extension at 72°C. A subsaturating number of cycles (10 to 15) allowed a semiquantitative analysis between each treatment. Dilutions of PCR products were visualized by ethidium bromide staining after agarose gel electrophoresis, and comparisons were made at the same dilution for each treatment. PCR for actin was for the same number of limiting cycles and at the same dilution.

DNA constructs. A fragment (318 nucleotides) of the human IL-8 promoter (from bases −273 to +45) was obtained by PCR of genomic DNA (20, 32). This fragment, which contained an NFκB consensus binding site at nucleotides −81 to −72, was ligated to the pGL basic luciferase expression vector (32). A mutated IL-8 promoter in which 1 nucleotide essential for NFκB binding at nucleotide −79 was changed from G to A was designed (32).

The viral homolog of IκB encoded in the A238L gene of the ASFV BA71V strain was cloned by PCR with primers flanking the coding sequence for this open reading frame. The PCR product was cloned into the eukaryotic expression vector pcDNA3 (Invitrogen), and this plasmid was designated pA238L.

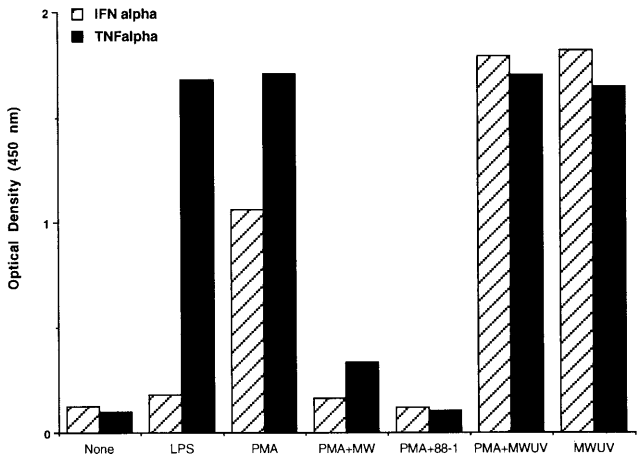

FIG. 1. Inhibition of PMA-stimulated IFN-α and TNF-α secretion in ASFV-infected alveolar macrophages. Alveolar macrophages were treated as indicated for 16 h, and supernatants were assayed by ELISA for IFN-α and TNF-α. Bars represent macrophage supernatants with no treatment (None) or treated with LPS at 10 μg/ml (LPS) or 100 nM PMA (PMA), cells stimulated with PMA and infected with ASFV Malawi isolate for 16 h (PMA+MW) or with PMA and ASFV OUR T/88-1 isolate (PMA+88-1), cells treated with PMA and infected with inactivated ASFV Malawi (PMA+MWUV), and cells infected with inactivated ASFV Malawi alone (MWUV). For IFN-α, 1.0 U at optical density at 450 nm is equivalent to 500 U of IFN-α per ml, and for TNF-α, 1.0 optical density unit is equivalent to 50 pg of TNF-α per ml when each is compared with known standards.

Transient transfection and luciferase assay. For transfections, porcine kidney cells, clone PK15-15 (4), were plated at 50 to 70% confluence in 150-cm^2 flasks, and 5 μg of IL-8 promoter-luciferase expression plasmid was added with Transfectam (Promega). After 16 h, cells were stimulated with PMA and either simultaneously infected with a tissue culture-attenuated isolate of ASFV (Uganda) (13) or cotransfected with pA238L, the expression vector encoding the IκB homolog from ASFV (BA71V) (36). Luciferase activity was assayed 4 h after PMA stimulation by homogenization in cell lysis buffer (Promega) followed by incubation of an aliquot of the cell extract with the luciferase substrate: 0.5 mM luciferin–0.27 mM coenzyme A–0.5 mM ATP–33.3 mM dithiothreitol–0.1 mM EDTA in Tricine buffer (Promega).

EMSA. PK15-15 cells were transfected with the eukaryotic expression plasmid pcDNA3 containing the viral IκB homolog, A238L (pA238L). After 18 h, cells were cultured with or without PMA (100 nM) for 4 h, and nuclear extracts were analyzed for NFκB binding activity by EMSA. In control experiments, nuclear extracts were prepared from nontransfected PK15-15 cells cultured either with or without PMA for 4 h. The binding of nuclear proteins to ^{32}P end-labelled oligonucleotide probes was analyzed on a 4% polyacrylamide gel in 0.5× Tris-borate-EDTA buffer. The same amount of protein from each nuclear lysate was loaded in each lane. The probes used were (i) a 24-mer double-stranded oligonucleotide containing the κB motif (shown in boldface) from the human IL-8 promoter from nucleotides −89 to −66 (20) (dGCAAATCG**TGGAATTTCC**T CTGAC), (ii) a 24-mer oligonucleotide with a mutation in the κB motif of G to A at nucleotide −79 (dGCAAATCG**TGAAATTTCC**TCTGAC), and (iii) an AP1 consensus site oligonucleotide (Promega). Where indicated, unlabelled competitor NFκB wild-type oligonucleotide was added to the binding reaction mixture for 20 min before addition of ^{32}P-labelled DNA.

RESULTS

ASFV inhibits PMA-stimulated IFN-α and TNF-α secretion from infected macrophages. IFN-α and TNF-α were undetectable in the media for untreated alveolar macrophages after 16 h in culture (Fig. 1, None). Addition of lipopolysaccharide (LPS) to macrophages stimulated secretion of TNF-α, but there was little IFN-α produced (Fig. 1, LPS). When cells were stimulated with PMA for 16 h, both IFN-α and TNF-α were detected at high levels in the conditioned medium (Fig. 1, PMA). Significantly, when macrophages were stimulated with PMA and at the same time infected with ASFV (either Malawi

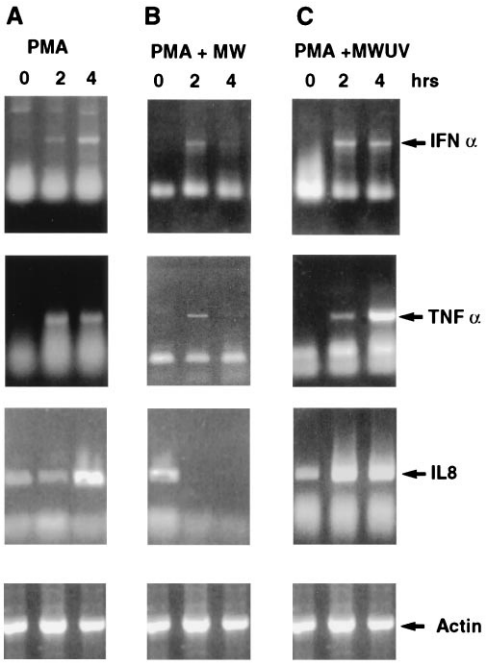

FIG. 2. PMA-induced transcription of antiviral proinflammatory cytokines is inhibited by viable ASFV but not by inactivated ASFV. Shown is analysis of cytokine mRNA levels in alveolar macrophages treated with 100 nM PMA (A), 100 nM PMA and ASFV Malawi (B), or 100 nM PMA and UV light-inactivated ASFV Malawi (PMA+MWUV) (C). RNA was isolated 0, 2, and 4 h after treatment and reverse transcribed into cDNA, and IFN-α, TNF-α and IL-8 mRNAs were semiquantitated by 10 to 15 cycles of PCR using cytokine-specific primers so that products were below the saturation stage of amplification. DNA products were separated by electrophoresis on a 2% agarose gel and visualized with ethidium bromide. Equal RNA was amplified for each sample as indicated by actin PCR shown below.

or OUR T/88-1 isolates) there was little IFN-α or TNF-α detected in the conditioned media after 16 h (Fig. 1, PMA+MW and PMA+88-1). The ASFV-mediated inhibition of IFN-α and TNF-α secretion was not altered by the addition of 3-formyl rifampin or cytosine arabinoside, inhibitors of early and late viral gene expression, respectively (26). Thus, the virus-induced inhibition was not dependent on new RNA or DNA synthesis. Moreover, the PMA-stimulated IFN-α and TNF-α secretion was not inhibited by infection of the cells with UV light-inactivated virus (Fig. 1, PMA+MWUV). In fact, UV light-inactivated virus alone stimulated IFN-α and TNF-α secretion (Fig. 1, MWUV). This may be explained by either inactive virion or virion components stimulating IFN-α and TNF-α secretion after binding to the surface cells, as has been shown for a variety of other viruses, or after being taken up by cells.

PMA-induced gene transcription of proinflammatory cytokines is inhibited by ASFV. RNA was isolated from macrophages incubated with either PMA or with PMA plus ASFV for 0, 2, or 4 h. RNA was reverse transcribed with avian myeloblastosis virus reverse transcriptase, and cDNAs for specific cytokines were amplified by PCR. PMA was a potent activator of IFN-α, TNF-α, and IL-8 transcription, and mRNA levels increased within 2 to 4 h (Fig. 2A). PCR for actin under limiting cycles showed similar amount of products in each lane for each sample. When macrophages were stimulated with PMA, and infected with ASFV, there was a small increase in transcription of IFN-α and TNF-α after 2 h, but this was suppressed by 4 h of viral infection (Fig. 2B), and so it is clear that ASFV inhibited the PMA-induced transcription of these cytokines very soon after infection (2 to 4 h). IL-8 mRNA levels were initially high and were suppressed by 2 h of infection (Fig. 2B). As might be predicted from the ELISA study, when macrophages were infected with UV light-inactivated ASFV and stimulated with PMA, IFN-α, TNF-α, and IL-8 gene transcription increased compared with PMA alone (Fig. 2C). The ability to inhibit PMA-induced secretion of these cytokines therefore resides in the live virus, and this activity is destroyed by UV light.

ASFV induces the secretion and transcription of TGF-β from infected macrophages. In order to determine whether the observed ASFV-mediated inhibition of proinflammatory cytokine secretion was restricted to selected cytokines or reflected a more general or nonspecific inhibition, TGF-β was measured in the supernatant of infected, noninfected, or PMA-stimulated macrophages (Fig. 3A). TGF-β secretion was undetectable from untreated alveolar macrophages (Fig. 3A, none). When the cultures were stimulated for 16 h with LPS or PMA, TGF-β levels significantly increased (Fig. 3A, LPS and PMA). Infection of macrophages with either live viable or UV light-irradiated ASFV in the absence of PMA resulted in similar levels of secreted TGF-β as in cells stimulated with PMA (Fig. 3A, MW, OUR T/88-1, and MWUV). Incubation of cells with both virus and PMA did not increase TGF-β secretion above that seen for either individually. The increase in TGF-β levels was not inhibited by 3-formyl rifampin or by cytosine arabinoside. The observations on the protein level were confirmed at the transcriptional level. RNA isolated from macrophages was reverse transcribed as above, and the single-stranded cDNA was amplified by PCR with primers for TGF-β. TGF-β mRNA levels were initially low in freshly plated macrophages (Fig. 3B). On stimulation with ASFV alone, TGF-β mRNA levels rose 10-fold after 2 to 4 h and then returned to normal 16 h after infection. A similar increase was also seen when macrophages were stimulated with PMA alone or when macrophages were infected with UV light-inactivated ASFV alone (data not shown). Importantly, these results show that ASFV does not downregulate transcription of all cytokine genes and that the effect is specific to proinflammatory cytokines.

Inhibition of proinflammatory cytokine transcription involves the NFκB pathway. The previous results showed that there is a decrease in transcription and translation of mRNA for proinflammatory cytokines. Although this may be due to increased turnover, we looked to see whether it was due to decreased transcription, possibly through a mechanism common to the genes for IFN-α, TNF-α, and IL-8 and based on the transcription factor NFκB. A series of experiments analyzing the transcription from an NFκB-containing promoter element and a reporter gene was carried out. The human IL-8 promoter, containing 318 bp upstream of the transcriptional initiation site (from bases −273 to +45) and a NFκB binding site, was cloned upstream of a luciferase reporter gene. This construct was transfected into porcine kidney (PK15-15) cells which had been shown by RT-PCR to express the proinflammatory cytokine IL-8 when stimulated with PMA (data not shown). After transfection overnight, cells either remained untreated or were stimulated for 4 h with PMA and then scraped into cell lysis buffer, and the homogenate was assayed for luciferase activity. Luciferase activity was detected without PMA treatment of transfected cells, probably reflecting the high level of cell proliferation in this cell line (Fig. 4, none). When cells were stimulated with PMA, twice as much luciferase activity was produced as with unstimulated cells (Fig. 4, PMA). These results indicate that the human promoter functioned efficiently in the heterologous porcine cells and was

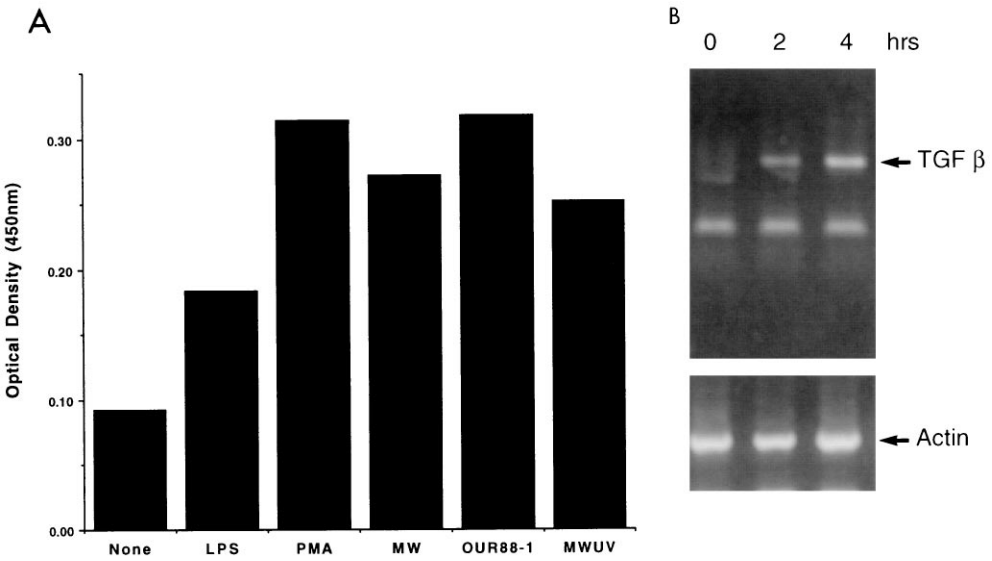

FIG. 3. Induction of TGF-β secretion and transcription from ASFV-infected alveolar macrophages. (A) Supernatants from alveolar macrophages infected with live ASFV and UV light-inactivated ASFV were collected after 18 h and assayed with a TGF-β-specific ELISA. Bars represent alveolar macrophages that were untreated (None); treated with 10 μg of LPS per ml for 16 h (LPS) or with 100 nM PMA for 16 h (PMA); or infected with ASFV Malawi alone (MW), ASFV OUR T/88-1 alone (OUR88-1), or UV light-inactivated ASFV (MWUV). A TGF-β standard of 1.2 ng/ml gave an optical density reading at 450 nm of 1.0. (B) RNA was isolated from alveolar macrophages infected with ASFV Malawi for 0, 2, or 4 h. TGF-β mRNA levels were analyzed by RT-PCR followed by agarose gel electrophoresis and staining with ethidium bromide. Equal RNA in each lane is indicated with actin PCR as a control. Similar increases were obtained after addition of 100 nM PMA or infection with inactivated ASFV (data not shown).

inducible by PMA. A mutant IL-8 promoter-luciferase construct with a single point mutation in the binding site for the transcription factor NFκB was used as a control in similar transfection experiments. This mutant construct failed to express luciferase activity with or without PMA stimulation (Fig. 4, mutant), indicating that NFκB binding to the IL-8 promoter element was essential for gene expression. Importantly, cells transfected with the authentic NFκB-containing IL-8 promoter plasmid and then infected for 4 h with the attenuated strain of ASFV (Uganda) showed greatly reduced amounts of luciferase activity (Fig. 4, ASFV), even when cells were stimulated with PMA at the same time as infection with ASFV (Fig. 4, PMA+ASFV). These experiments were repeated four times with the same results.

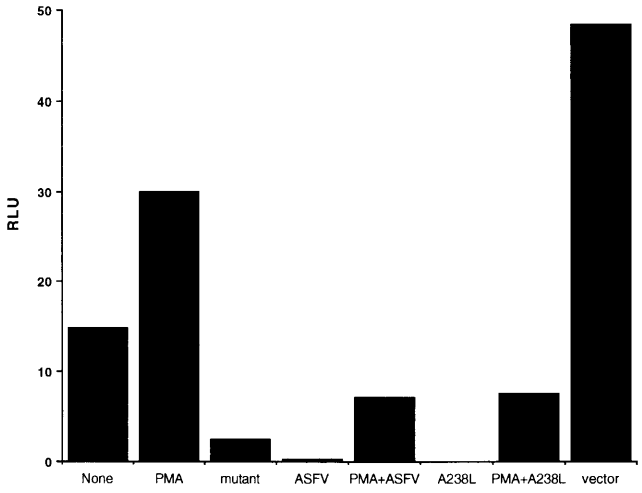

FIG. 4. Inhibition of NFκB-activated gene expression by ASFV infection and by the cloned virally encoded IκB homolog A238L. PK15-15 cells transfected with the human IL-8 promoter, containing an NFκB recognition sequence, cloned upstream from a luciferase reporter cDNA were assayed for luciferase activity (expressed as relative light units [RLU]). Transfected cells were assayed with no treatment (None) or after stimulation with PMA for 4 h to activate NFκB (PMA). Transfection of a mutant promoter in which the IL-8 κB motif at nucleotides −82 to −70 (GTG**G**AATTTCCTC) was changed to GTG**A**AATTTCCTC was used to show that all activity was dependent on NFκB binding (mutant). Cells transfected with the wild-type IL-8 promoter construct were infected for 4 h with ASFV (ASFV) or infected with ASFV and stimulated with PMA for 4 h (PMA+ASFV). The IL-8 promoter construct was cotransfected with pA238L (A238L) or cotransfected pA238L and stimulated with PMA for 4 h (PMA+A238L). Transfection of the empty pcDNA3 parental vector with the IL-8 promoter construct was included as a control (vector).

An ASFV homolog of IκB, encoded by A238L, is responsible for NFκB inhibition. The recent publication of the complete sequence of ASFV (36) suggested that the simplest and most logical interpretation of this inhibition of NFκB activity by ASFV resided in the virally encoded homolog of IκB, encoded by the gene A238L. This gene is 20% identical and 41% homologous at the amino acid level to the porcine IκB protein (see Fig. 6). To prove this, the cDNA for A238L was cloned into an expression vector, pcDNA3, and cotransfected simultaneously with the IL-8 promoter-luciferase reporter construct into PK15-15 cells. These doubly transfected cells had reduced luciferase activity (Fig. 4, A238L), even when stimulated with PMA (Fig. 4, PMA+A238L) in comparison with cells not transfected with A238L. In contrast, significant levels of activity were obtained when the parent vector used for expressing the A238L gene of ASFV (pcDNA3) was cotransfected with the IL-8 promoter-reporter construct and stimulated with PMA (Fig. 4, vector), showing that competition for transcription factors binding to the A238L expression vector did not nonspecifically reduce the activity of the luciferase reporter gene. The results of this reporter gene experiment indicated that the viral homolog of IκB (A238L) decreased NFκB-activated gene expression and was responsible for the observed proinflammatory cytokine inhibition.

A238L inhibits the binding of NFκB to DNA. EMSA of nuclear extracts from transfected and nontransfected cells was used to investigate the effect of the viral IκB homolog, A238L,

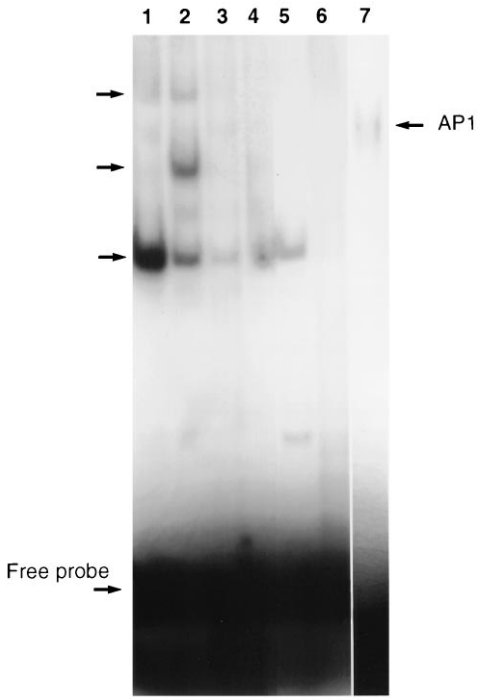

FIG. 5. EMSA of nuclear extracts from PK15 cells expressing the viral IκB homolog A238L. Analyses were performed with an oligonucleotide containing the κB motif from the IL-8 promoter from nucleotides −89 to −66 (lanes 1 to 4 and lane 6) or an oligonucleotide containing a single change from G to A at nucleotide −79 in the κB site (lane 5) or an AP1 consensus oligonucleotide (lane 7). Nuclear extracts from the following conditions are shown: nonstimulated PK15-15 cells (lane 1), PMA-stimulated PK15-15 cells (lane 2), PK15-15 cells transfected with pA238L (lane 3), PMA-stimulated PK15-15 cells transfected with pA238L (lane 4), PMA-stimulated PK15-15 cells incubated with the mutant oligonucleotide (lane 5), PMA-stimulated PK15-15 cells preincubated with unlabelled oligonucleotide (lane 6), and PK15-15 cells transfected with A238L and incubated with AP1 oligonucleotide (lane 7). Arrows show three NFκB protein complexes binding to the specific recognition site. Control showing the AP1 protein is indicated. Free probe is seen at the bottom of the gel.

on NFκB protein binding to an oligonucleotide containing the IL-8 κB DNA sequence. The ability of nuclear proteins from nontransfected PK15-15 cells to bind to the IL-8 κB binding site in control experiments is shown in Fig. 5. In normal, unstimulated cells, one protein bound to the ^{32}P-labelled oligonucleotide containing the IL-8 κB site, seen as a band shifted in molecular weight (Fig. 5, lane 1). Cells stimulated with PMA showed this protein and several other complexes of higher molecular weight (Fig. 5, lane 2) as has been reported previously for NFκB proteins binding to this region of the IL-8 promoter (32). When nuclear extracts from cells transfected with the viral IκB homolog were assayed, a much-reduced weak band at the lower molecular weight and no higher-molecular-weight bands were seen both with and without PMA stimulation (Fig. 5, lanes 3 and 4). Control experiments were performed to show that the shifted bands seen in untransfected cells were due specifically to NFκB. A single point mutation in the κB site (see Materials and Methods) much reduced the band seen at the lower molecular weight and abolished the higher-molecular-weight bands seen in nuclear extracts from PMA-treated, nontransfected cells (Fig. 5, lane 5), showing that proteins seen with the wild-type probe were specifically binding to the κB site. Also, preincubation with nonlabelled competitive DNA containing the NFκB binding site abolished the mobility shift seen in nontransfected cells (Fig. 5, lane 6). In order to show that lack of DNA binding in the transfected cells affected only NFκB binding to its specific oligonucleotide, an AP1 consensus DNA sequence was used to show that binding of this nuclear protein was unaffected in cells expressing A238L (lane 7). These results indicate that NFκB binding to its specific DNA promoter site is greatly reduced in cells transfected with the A238L IκB homolog.

DISCUSSION

This paper describes a novel virus immune evasion strategy. We show that infection of porcine alveolar macrophages with ASFV leads to an almost complete shutdown of proinflammatory cytokine secretion. This correlated with a decrease in transcription of TNF-α, IFN-α, and IL-8 mRNA as measured by RT-PCR. The inhibition of transcription occurred between 2 and 4 h after infection, indicating a very early disruption of macrophage inflammatory defenses and the potent activity of the virus against early induction of interferons. The action of ASFV on expression of TGF-β, a cytokine which inhibits the respiratory burst and inflammatory responses, was also investigated. ASFV increased transcription of TGF-β, suggesting a specific inhibition of proinflammatory, but not anti-inflammatory, cytokines by the virus. The observed increase in TGF-β synthesis argues against protein synthesis shutdown as a mechanism for downregulation of proinflammatory cytokine transcription. Unlike in vaccinia virus infection, in which host protein synthesis is inhibited, biosynthetic labelling studies have shown that total protein levels in cells are not changed 2 to 8 h after ASFV infection (23a).

Rapid proinflammatory cytokine transcription is activated by the transcription factor NFκB (3). In order to test whether ASFV infection resulted from inhibition of NFκB activity, an NFκB-containing promoter from the human IL-8 gene was placed upstream of a luciferase reporter gene (32). This promoter element was functional in transfected cells and responded to PMA stimulation, resulting in the increased expression of luciferase activity. The activity was inhibited by infection of cells with ASFV. A single gene from ASFV, A238L, encoding the IκB homolog, was shown to inhibit the NFκB reporter assay, strongly suggesting that this viral gene blocks NFκB activation. The observation by EMSA that the A238L gene product specifically inhibited binding of NFκB to its DNA binding site further supports an interaction of this viral protein with NFκB.

NFκB is a dimer of two heterologous proteins (typically p65 and p50) held in an inactive complex with an endogenous inhibitor, IκB, in the cytoplasm. After cell activation, IκB is phosphorylated and subsequently degraded by cytoplasmic proteosomes. The released NFκB now translocates to the nucleus, where it binds to regulatory enhancer elements upstream from the transcriptional initiation site of proinflammatory cytokine genes. Phosphorylation of IκB is important for the activation of NFκB but does not induce IκB dissociation from the inactive NFκB complex. Selective degradation of phosphorylated IκB is known to be a critical factor for NFκB activation. The ASFV gene A238L has 21% identity and 40% homology at the amino acid level to porcine IκBα, one of the members of the family of inhibitors of NFκB which binds to it in the cytoplasm. An alignment of porcine IκBα, referred to as ECI-6 (9), and the ASFV gene A238L is shown in Fig. 6. Both NFκB and IκB proteins contain ankyrin sequence motifs in tandem arrays that are involved in protein-protein interaction (19). Porcine IκBα contains five repeats of the ankyrin consensus sequence (9). The alignment in Fig. 6 shows that the ASFV IκB homolog protein has deletions in the first two of these

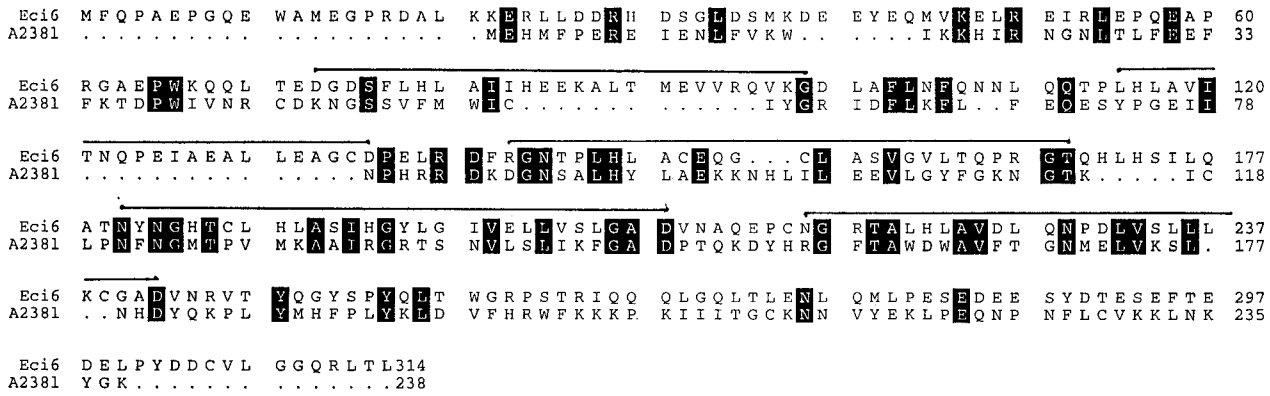

FIG. 6. Alignment of porcine IκBα (ECI-6) and the ASFV IκK homolog (A238L). Shaded residues are identical between ECI-6 and porcine IκBα, and lines are shown above the five repeats of the ankyrin consensus sequence in IκBα.

areas and about 35% conservation of the three other ankyrin repeat domains. One mechanism of viral IκB-A238L action could be to bind to cellular NFκB through these regions in the same way as its cellular counterpart. Three potential phosphorylation sites in IκBα have been described previously (9), including a casein kinase II site (Ser-32), a tyrosine phosphorylation site (Tyr-42), and a protein kinase C phosphorylation site (Ser-262). The A238L sequence lacks each of these phosphorylation sites (Fig. 6). The absence of potential phosphorylation sites may indicate that the homolog fails to respond to stimulation of cells and retains the ability to bind to and inactivate NFκB. Alternatively, it has been shown that free IκB can dissociate a preformed NFκB DNA complex (37). If vIκB localizes to the nucleus, then it could have a direct inhibitory effect on transcription. The rapid (4-h) reduction of proinflammatory cytokine mRNA levels suggests that this may be true. Further experiments will determine the mechanism of action of A238L through production of anti-vIκB antibodies to localize the viral gene product after cell activation and characterization of proteins that bind to it. Subcellular localization of NFκB may be altered after A238L expression.

Not only may vIκB-A238L be responsible for the downregulation of the proinflammatory cytokines seen after virus infection, but it also may inhibit a range of macrophage NFκB-activated genes. For example, NFκB activates nitric oxide synthase, which produces nitric oxide, a messenger in several antiviral mechanisms. Other important macrophage molecules controlled by NFκB include major histocompatibility complex classes I and II, adhesion molecules, complement factors, and acute-phase proteins. In contrast to ASFV, some other viruses, such as influenza virus (23), human immunodeficiency virus type 1, cytomegalovirus, and simian virus 40, and also other large DNA viruses such as adenovirus, Epstein-Barr virus, and herpes simplex virus (3) upregulate host cell cytokines through an NFκB pathway, and they may use NFκB to transcribe their own genes. This work reveals that ASFV has developed a novel mechanism to inhibit proinflammatory, antiviral cytokine production, by using a virally encoded protein to inhibit NFκB-activated transcription. By inhibiting a central transcriptional control point, a single viral protein can counteract the action of several cytokines, in contrast to poxviruses, in which several proteins are needed to encode receptor homologs to inhibit individual cytokines.

The expression of a viral IκB homolog may account for some of the pathological changes seen in ASFV infection. Inactivation of NFκB in mice has been shown to result in lymph node disorganization, impaired cellular immunity, and multifocal mixed inflammatory cell infiltration as well as the mice being less able to clear pathogens (27, 33). Changes in macrophage proinflammatory cytokine expression may also contribute to blood vessel leakage and hemorrhage through their modulation of endothelial cell function. Finally, the employment of this ASFV-evolved strategy for the manipulation of proinflammatory cytokine expression might find application in human medicine when it is desirable to downregulate levels of these cytokines, such as in inflammatory diseases.

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